REMARKS

Applicant begins by thanking the Examiner for her helpful suggestions.

I. Status of the Claims and Support for the Amendments

Claims 28, 48, 49, and 51–53 are currently amended and new claim 54 is added.

Claims 28–30 and 48–54 are currently pending.

Applicant believes that support for the current amendments to the claims is implicit in the claims as pending prior to the amendment. Support for new claim 54 is found in previously pending claim 28. Further, applicant does not believe that any of the amendments made to claims 28, 48, 51, or 52 are narrowing amendments. Rather, the amendments to these claims either correct typographical errors or merely clarify and make explicit that which was previously implicit in the claim. Moreover, taken together with new claim 54, the amendment to claim 53 does not result in a narrowing of the claim set. Rather, claims 53 and 54 now represent two species of generic claim 28.

Notwithstanding the foregoing, if any claim scope is deemed to have been removed by the current amendments, Applicant explicitly reserves the right to pursue such material in one or more continuation or divisional applications.

II. Objection to the claims

Claim 53 is objected to as allegedly not further limiting claim 28. The Examiner indicates that the limitation "4:1 ratio by weight" is recited in both claim 28 and claim 53. Applicant responds as follows.

Currently amended claim 53 and new claim 54 are now respectively drawn to methods pertaining to the use of compositions comprising compounds of formulas VI and I and formulas VI and III. Accordingly, Applicant believes that these claims both *further* limit claim 28, which is drawn to methods pertaining to the use of compounds of formulas VI and (I *or* III).

Accordingly, Applicant believes that the objection to claim 53 has been overcome and may now properly be withdrawn.

III. Rejection under 35 U.S.C. §112, second paragraph

Claims 28–30 and 48–53 are rejected under 35 U.S.C. §112, second paragraph as allegedly being indefinite.

A. Claim 28 is rejected under 35 U.S.C. §112, second paragraph for allegedly being indefinite for its use of the phrases "*carcinoma of an unknown primary*" and for allegedly not being clear as to whether "the patient needed the recited treatment." Applicant responds as follows.

In response to this objection claim 28 has been modified in accordance with the Examiner's suggested amendments. Consequently, Applicant believes that this rejection of claim 28 has been overcome and may now properly be withdrawn.

B. Claim 48 is rejected under 35 U.S.C. §112, second paragraph as allegedly being indefinite because of its use of the phrase "*[t]he method of composition of claim 28*". In response, this phrase has been amended to recite "*[t]he method of claim 28*" as suggested by the Examiner. Accordingly, Applicant believes that this rejection has been overcome and may now properly be withdrawn.

C. Claim 49 is rejected under 35 U.S.C. §112, second paragraph as allegedly being indefinite for the use of the phrase "*or pharmaceutically acceptable salts thereof*" when referring to a compound of formula IV, I, and III. In response, in each place this phrase was used, it has been replaced with the phrase "*or a pharmaceutically acceptable salt thereof*", in accordance with the Examiner's suggestion.

In view of these amendments, Applicant believes that this rejection of claim 49 has been overcome and may now properly be withdrawn.

D. Claims 51 and 52 are rejected under 35 U.S.C. §112, second paragraph as allegedly being indefinite for their use of the phrase "*or pharmaceutically acceptable salts thereof*". In response applicant has replaced, in each claim, the offending phrase with the phrase "*or a pharmaceutically acceptable salt thereof*", as suggested by the Examiner.

Consequently, Applicant believes that this rejection of claims 51 and 52 has been overcome and may now properly be withdrawn.

IV. Rejection under 35 U.S.C. §103

Claims 28–30 and 48–53 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable for being obvious over *Recent Advances in Chemotherapy*, Adam, D. ed. Munich: Futuramed, 1992 (referred to herein as "Adam *et al.*"), which is described in the instant Application at page 5, third full paragraph (Applicant points out that "Adam" is not the author of the reference referred to as Adam *et al.*, rather Adam is one of the editors of the cited document. Further, as noted below and as specifically requested by the Examiner, a full copy of the cited portion of this reference is enclosed herewith).

The Examiner alleges that:

> Adam *et al.* teach using A10 (compound III) and AS2-1 (compound IV) to treat cancer.
>
> The instant independent claim differs from Adam *et al.* in reciting the use of the compound of Formula III and the compound of Formula IV together, a ratio of the compound of Formula IV and the compound of III of 4:1 and that the total combined concentration of the compound of Formula IV and the compound of Formula III is from 70 mg/ml to 150 mg/ml. Dependent claims recite specific dosages, rates of infusion and combined concentrations of compounds.
>
> However, in the absence of a showing of unexpected results no unobviousness is seen in combining the compound of Formula III and the compound of Formula IV together, since each is taught to be useful to treat cancer. Furthermore, once the

> usefulness of a composition is taught, it is within the skill of the artisan to determine the optimum dosages, rates of infusion and combined concentration of compounds.

See, Office Action pages 3 and 4. Applicant respectfully traverses.

MPEP § 2143 sets out the following requirements that must all be met to establish a *prima facie* case of obvious:

> To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.
>
> The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, not in the applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991)

MPEP §2143 (emphasis added).

Applicant asserts that Adam *et al.* fails to meet all of these requirements. Taking the requirements in reverse order, Applicant contends that Adam *et al.* fails to teach or suggest the *high* infusion rate required by the currently pending claims (*i.e.* about 100 ml/hour to about 400 ml/hour, *see* line 18 of claim 28). Because of their typically toxic nature, chemotherapeutic agents are generally given at as low a dose and as slowly as possible; thus one of the results discovered by the Inventor was that administration of the compounds according to the claimed methods unexpectedly reduced drug toxicity.

For example, as described in the Specification:

> administration of a solution comprising the hydrolysis products of 3-phenylacetylamino-2,6-piperidinedione at low infusion rates of from 2.5 mL/h to 84 mL/h frequently results in an elevation in levels of waste products in plasma. An exemplary waste product so elevated is uric acid. This elevation interferes with treatment by requiring either a decrease in the dose or an interruption in the treatment to administer additional drugs, for example, Allopurinol, to decrease the level of the waste product, for example, uric acid.

Page 6, lines 21–26 of the Specification (emphasis added). In contrast, one of the advantages of the currently claimed method is that

> [t]he high flow rate of antineoplaston AS2-1[, a composition comprising a 4:1 ratio of phenylacetate, formula IV, and phenylacetylglutamine, formula I,] infusion and the high concentration of antineoplaston AS2-1 produce a diuretic effect. The diuretic effect is beneficial to the patient in preventing fluid overload from large infusion volumes and in providing a mechanism for elimination of waste products which can otherwise accumulate in the body, as described above.

Page 20, lines 6–9, of the Specification (emphasis added).

Thus, the instant application teaches two unexpected advantages resulting from the high infusion rates taught therein. First, it allows for a higher plasma level of the active compounds, which in turn increases the penetration of the active compound into the tumor. Second, the "diuretic effect" produced by these methods both prevents fluid overload and eliminates toxic waste products, as well as eliminating the need for treatment with additional drugs, like Allopurinol. Moreover, there is nothing in Adam *et al.* that teaches or suggests the use of high infusion rates, much less the unexpected advantages of using these high infusion rates. These advantages are demonstrated for the first time in the instant application.

It is Applicants position that Adam *et al.* taken either alone or in view of what was known by those of ordinary skill in the art provides no motivation to modify the teachings of Adam *et al.* to both treat patients with a combination of a compound of Formula III and Formula IV and to provide these compounds at a high infusion rate.

The rejection states that "it is within the skill of the artisan to determine the optimum dosages, rates of infusion, and combined concentration." The knowledge of those of ordinary skill in the art "teaches away" from increasing the dosage or infusion rate of a chemotherapeutic agent when the current infusion rate is toxic. In contrast, the instant invention shows that very

high infusion rates, rather than exacerbating toxic effects, instead ameliorate and/or prevent them. Thus, the instant invention teaches away from conventional wisdom.

In view of the foregoing arguments, Applicant believes that the rejection of the claims as being obvious under 35 U.S.C. §103(a) in view of Adam *et al.* has been overcome and may now properly be withdrawn.

V. Response to request for cited document

As requested, Applicant provides herewith the full citation for Adam *et al.* and a copy of the cited portion thereof. The reference for the cited document is "Recent Advances in Chemotherapy: Anticancer Section" *Proceedings of the 17th International Congress of Chemotherapy*, Berlin, 1991, Dieter Adam, Thomas Büchner, and Ethan Rubinstein, Eds, Futuramed Publishers, Munich, Germany, 1992. The cited specific portion cited is Session (42) *Novel Differentiation Inducers*.

VI. Conclusions

In view of the foregoing Amendments and Remarks, Applicant believes that all objections to and rejections of the instant application have been overcome and that the application is now in condition for immediate allowance. Accordingly, Applicant respectfully requests favorable reconsideration of the instant Application and issuance of a Notice of Allowance therefor.

The Examiner is invited to contact the undersigned patent agent at (713) 787-1589 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,

/Matthew Madsen

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Date: January 20, 2004

RECENT ADVANCES IN CHEMOTHERAPY

Anticancer Section

Proceedings of the 17th International Congress of Chemotherapy
Berlin, 1991

Edited by Dieter Adam, Thomas Büchner and Ethan Rubinstein

Futuramed Publishers, Munich, Germany

Contents

Session
(42)

Novel Differentiation Inducers

Novel Differentiation Inducers

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It is proposed that in addition to the immune system, the human organism has a second defense which is the Biochemical Defense System (BDS) of differentiation inducers. The BDS provides defense against defective cells and does not involve destruction but the reprogramming of cells through the induction of differentiation. Errors in cell programming may lead to such diverse disorders as cancer, benign tumors, AIDS and Parkinson's disease. Chemical components of the BDS are peptides, amino acid derivatives and organic acids defined as antineoplastons (AN). The known mechanism of action of AN includes inhibition of incorporation of glutamine, specific intercalation with DNA and inhibition of methylation of nucleic acids. Phase I trials indicated a lack of significant toxicity. Phase II trials in advanced prostate cancer and astrocytoma documented a significant number of objective responses.

For more than a century, the immune system has been regarded as the main defense of the body. From the initial study of the protection against infectious disease, the science of immunology has expanded greatly to include, among others, immunotherapy of cancer.

The basis of the immune reaction is the response of the organism to antigenic challenge and the main purpose is the protection of the body against external invaders, such as microorganisms. Chemical components of the immune system are proteins and the mechanism of defense is the destruction of an invading agent.

Looking from a different perspective, the research on growth factors and growth inhibitors during the last twenty years indicates the possibility of the existence of another defense system in our body. This is the system of differentiation inducers for which I proposed the working name of Biochemical Defense System (BDS) (1). This system protects the organism against the enemy within the body. The main purpose is no longer the defense against the microorganisms, but defense against defective cells. Chemical components of this system are peptides, amino acid derivatives and organic acids defined as antineoplastons (AN) (2). The mechanism of defense is based not on destruction, but on the reprogramming of defective cells through induction of differentiation.

The research on AN began in 1967 (3,4). Initially, the work concentrated on the isolation of peptides which exist in the blood of healthy people and are deficient in cancer patients. Due to the small amount of raw material available for the study, in the following years AN were isolated from urine, instead of blood. In 1980, the structure of the first AN was identified and reproduced synthetically (5).

AN are divided into two groups. One group contains compounds which have a wide spectrum of activity and includes AN A1, A2, A3, A4, A5, A10, AS2-1 and AS2-5. AN A1, A2, A3, A4 and A5 contain peptides isolated from urine and AN A10, AS2-1 and AS2-5 are the synthetic products. In addition to this first group, there are AN which are active against a single specific type of neoplasm, such as AN H, L and O.

It is postulated that under the influence of AN, cancer cells change into differentiating cells, which will undergo terminal differentiation converting them into senescent cells committed to die. From the stage of uncontrolled proliferation, the cells will enter controlled proliferation followed by irreversible senescence and cell death. AN exert their activity from G_1 to early S phase in the cell cycle.

Three known mechanisms of action of AN include: 1) inhibition of incorporation of glutamine into the growing polypeptide chain, 2) specific intercalation with DNA and 3) inhibition of methylation of nucleic acids. AN AS2-1 and AS2-5 work according to the first mechanism, AN A10 according to the second and AN A2, A3 and A5 inhibit methylation of

RNA and DNA in neoplastic cells. AN AS2-1 induces relative glutamine deficiency and substitutes glutamine with phenylacetylglutamine. Three possible mechanisms are postulated for the induction of terminal differentiation by AS2-1: deviation from the genetic code, modification of DNA bases and RNA editing (6). Details of the mechanism of action of AN A10 and its new analogues will be discussed by L.B. Hendry at this session. Two additional presentations by M. C. Liau and S.S. Lee will discuss the inhibition of methylation of nucleic acids by AN A2, A3 and A5 and the synergism of A5 and retinoic acid in the induction of differentiation in human promyelocytic leukemia.

The research on AN followed generally accepted stages of new drug development, beginning with preclinical studies and progressing through Phase I and II clinical trials. Phase I trials revealed a lack of significant toxicity and interesting objective responses, including complete and partial remissions. Twenty percent of the advanced cancer patients who participated in the Phase I trial of AN A2 survived free from cancer over five years. Promising results of the treatment were observed in urological cases of adenocarcinoma of the prostate and transitional cell carcinoma of the bladder and in primary malignant brain tumors (7,8).

AN A2, A3, A5, A10 and AS2-1 were selected for Phase II clinical trials. One of these trials involved the use of AN AS2-1 capsules in the treatment of hormonally refractory cancer of the prostate (6). Out of 14 patients involved, complete remission was obtained in two patients and partial remission in three patients. Only two patients showed progression of the disease. The results of Phase II trials of AN A10 and AS2-1 infusions in astrocytoma will be discussed at the present session. An additional Phase II trial in high grade glioma is now in progress. Permission was obtained from the U.S. FDA to conduct a Phase II trial of AN A10 capsules in advanced breast cancer. In addition, clinical trials are now being conducted at the University of Kurume Medical School (Japan) and at the Medical Academy in Warsaw (Poland). The results of the treatment of malignant brain tumors and hepatoma at the University of Kurume will be presented at this session.

In addition to the treatment of cancer and benign tumors, there are other possible applications of antineoplastons. Quantitative determination of AN components in plasma and urine can be used for early diagnosis of cancer and in following the response to anticancer therapy (9,10). Extensive animal testing indicates the potential application of AN A10 in chemoprevention of lung and breast cancer and hepatoma (11-14). Phase II clinical trials are now being conducted in AIDS and asymptomatic HIV infection with initial interesting results (15). Laboratory and limited clinical data indicate the possible application of AN A10 in the treatment of parkinsonism and A5 in Parkinson's disease (16-18).

The lack of toxicity and the simplicity of clinical application and activity in the types of cancer which do not respond well to established clinical regimen makes these novel differentiation inducers attractive objects of future studies.

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Prevention of Drug-induced DNA Hypermethylation by Antineoplastons

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The methylation enzymes of neoplastic cells are altered by acquiring a cancer specific protein factor which enables these enzymes to assume a stable and active configuration. These abnormally active enzymes were responsible for causing DNA hypermethylation when DNA synthesis of HL-60 cells was disrupted by cytotoxic drugs which were devoid of noticeable effect on the methylation enzymes. Pretreatment of HL-60 cells with the purified active components of antineoplastons, which have been shown to antagonize selectively the cancer specific protein factor of methylation enzymes, prior to cytotoxic drugs eliminated drug-induced DNA hypermethylation. In view of the fact that DNA methylation plays a certain role on gene expression, it is anticipated that antineoplastons may prevent adverse effects arising from drug-induced DNA hypermethylation.

The methylation enzymes of neoplastic cells are uniquely altered by acquiring a cancer specific protein factor which enables these enzymes to assume a stable and active configuration (1). Thus, neoplastic cells possess an enzyme system locked in active state to carry out efficient methylation. The efficient methylation locked in place constitutes a major problem of cancer (2). Terminal differentiation is mediated through a critical process responsible for inducing DNA hypomethylation of the growth population (3). With methylation enzymes locked in active state, DNA methylation pattern can be efficiently reproduced to maintain neoplastic phenotype. Therefore, efficient methylation is a determining factor to perpetuate neoplastic growth. Precisely this efficient methylation capability of neoplastic cells is responsible for the observed DNA hypermethylation when DNA synthesis was disrupted by cytotoxic drugs (4). Methylation pattern once altered may lead to the emergence of cells with altered phenotypes, some of which may be troublesome, such as drug resistance and increased malignancy often attributable to the treatment with cytotoxic drugs. In order to prevent such troublesome complication, it is desirable to eliminate drug-induced DNA hypermethylation. We have previously demonstrated that the abnormal methylation enzymes of neoplastic cells could be effectively converted to the normal enzymes by antineoplastons which antagonized selectively the cancer specific protein factor of methylation enzymes (3). Once the abnormal methylation enzymes were corrected to become the normal enzymes, methylation activities could no longer keep in pace with synthetic activities, resulting in the production of hypomethylated nucleic acids and subsequent terminal differentiation. By the same reasoning, modulation of the abnormal methylation enzymes of neoplastic cells by antineoplastons may be useful to prevent drug-induced DNA hypermethylation. The present study was undertaken to explore this possibility.

The inhibition of DNA synthesis in cultured HL-60 cells by cis-DDP (cis-platinum diamine dichloride) and MTX (methotrexate) resulted in the production of hypermethylated DNA. Inhibitors were employed to give the inhibition of DNA synthesis above 82%. The level of induced DNA hypermethylation was proportional to the extent of the inhibition of DNA synthesis, ranging from 112% to 129%. In contrast, when DNA synthesis was inhibited to comparable degrees by nalidixate, DNA methylation remained at the control level. Thus, different inhibitors exert different influence on DNA methylation. When tested on the cancer isozyme MAT^{LT}, which was an altered enzyme of the ternary methylation enzymes consisting of MAT (methionine adenosyltransferase)-MT (methyltransferase)-SAHH (S-adenosylhomocysteine hydrolase), both cis-DDP and MTX showed absolutely no effect either in vitro or in vivo. Nalidixate, on the other hand, produced dose dependent inhibition of this enzyme in vitro

and significantly inactivated this enzyme in vivo as evidenced by the reduction of specific V_{max}. Unlike antineoplaston components which caused the K_m value to reduce greatly from 20 μM methionine of the cancer isozyme to 3 μM methionine of the normal isozyme, nalidixate caused only a slight reduction of the K_m value to 16 uM methionine. Therefore, mechanisms of inhibition by nalidixate and antineoplastons are apparently different. The inhibition by nalidixate appears to be mediated by nonspecific inactivation of the enzyme. In view of the fact that the inhibition of MAT ^{LT} was much less in comparison to the inhibition of DNA synthesis, e.g. 47% vs. 76%, factors other than the inhibition of MAT ^{LT} must be considered to play a significant role in the lack of effect to induce DNA hypermethylation by nalidixate. An additional inhibition of DNA methyltransferase by nalidixate is a possibility. Since the target of nalidixate inhibition of DNA synthesis is topoisomerase (5), simultaneous inhibition of DNA synthesis and DNA methylation can occur if topoisomerase is also involved in making methylation sites available for the methylation enzymes. Further studies are needed to clear these possibilities.

The treatment of HL-60 cells with the active components of antineoplastons purified as previously described (6) resulted in the production of hypomethylated DNA and NBT (nitroblue tetrazolium) positive cells. Antineoplaston components were employed to reduce methylation levels to 88-90% of the control and NBT+ cells above 80%. The pretreatment of HL-60 cells with antineoplaston components prior to cis-DDP and MTX effectively eliminated DNA hypermethylation induced by these drugs. Although the levels of DNA methylation were invariably higher in the presence of cytotoxic drugs, the levels of DNA methylation were nevertheless below that of the control. These results suggest that antineoplastons are useful to prevent drug-induced DNA hypermethylation and consequently adverse side effects arising from DNA hypermethylation. DNA hypermethylation can lead to inactivation of genes. The inactivation of genes involved in the activation of prodrugs can lead to the emergence of cells resistant to such prodrugs. The inactivation of suppressor genes can lead to the emergence of cells with increased malignancy. Such potentially troublesome adverse effects can be avoided if the abnormal methylation enzymes of neoplastic cells are first modulated by antineoplastons prior to the application of cytotoxic drugs. A major mechanism in the development of drug resistance is mediated through gene amplification. According to Adams and Burdon (7), the origin of DNA replication may start at a methylated site. The initiation proteins, however, effectively block rapid methylation of this origin site, which are removed at the end of S phase to undergo delayed methylation. Under metabolic blockade of DNA synthesis by cytotoxic drugs, the initiation proteins may be prematurely removed and the hemimethylated origin is quickly methylated to start illegitimate rounds of replication. Thus, if a cell possesses an efficient methylation capability like cancer cell, gene amplification may take place to a greater extent under metabolic blockade of DNA synthesis.

Agents causing DNA hypomethylation and DNA hypermethylation are likely to exert antagonistic effects. It is not surprising, therefore, to find that some cytotoxic drugs significantly reduced the ability of antineoplaston components to induce terminal differentiation. The combined application of antineoplastons and such cytotoxic drugs is, however, designed to reduce adverse effects of cytotoxic drugs rather than to increase the therapeutic efficacy. The purpose is, therefore, to utilize their antagonistic effects.

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Structure-activity Relationships, Molecular Modeling and Antitumor Activity of Phenylacetylamino-2,6-piperidinediones.

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Molecular modeling techniques developed in our laboratories which utilize degree of complementarity with DNA structure to predict biological activity were applied to the development of new analogs of the antitumorigenic compound, phenylacetylamino-2,6-piperidinedione (A10). The best fit into DNA as measured with energy calculations was p-OH-A10 which was capable of inserting between base pairs and forming hydrogen bonds bridging both DNA strands. Synthesis and biological testing of A10 analogs in various tumor cells revealed that p-OH-A10 was indeed the most active compound with up to 90% greater activity than A10.

In the process of screening fractions of human urine for antitumor activity in cell culture, a novel modified amino acid, 3-phenylacetylamino-2,6-piperidinedione (A10), was discovered. The structure of this compound, named A10 based upon the chromatographic fraction from which it was isolated, was confirmed by spectroscopic methods and independent synthesis (Burzynski et al., 13th Internat. Congr. Chemother., 1983, Vol. 17, P.S. 12.4.11-4). In subsequent studies, we showed that A10 fit between base pairs in partially unwound double stranded DNA and formed a single hydrogen bond between the imine proton of the piperidinedione ring and a phosphate oxygen of the deoxyribose-phosphate backbone. This study focused on the development of new analogs of A10 using a general molecular modeling technique developed in our laboratories (U.S. Patent 4,461,619). Briefly, this technique which can greatly aid in the design of compounds with improved activity relies on degree of fit into DNA to predict activity. We wish to emphasize, however, that the primary goal of this approach has been drug design and **not** to provide a framework to study physicochemical interactions with DNA or to infer that the mode of action of the compounds designed involves a direct interaction with DNA.

Computer modeling was conducted with Sybyl/Mendyl 5.3 software (Tripos Associates, St. Louis, Mo.) run on a Digital Microvax II Computer interfaced via Ethernet to an Evans and Sutherland PS390 computer equipped with Stereographics. A10, related analogs and partially unwound DNA were constructed from fragment libraries. Candidate analogs were individually docked between base pairs using monitor pairs to assess the strength and direction of potential hydrogen bonds. Inspection of the A10/DNA complex revealed that incorporation of a hydroxyl group in the para position (p-OH-A10) would enable formation of a second hydrogen bond thereby bridging both strands of DNA. Force field energy calculations measuring both

the electrostatic interactions due to hydrogen bonding and the van der Waals interactions resulting from steric contact were performed before and after docking of various A10 analogs. The change in energy resulting from insertion of each analog was measured (Table below).

ENERGY CHANGE IN KCAL RESULTING FROM INSERTION OF ANALOG INTO DNA

Compound	van der Waals	Hbond 1	Hbond 2	Total
p-OH-A10	-18.250	-21.653	-24.566	-64.469
o-Fluoro-A10	-18.675	-21.653	0.000	-40.328
p-Fluoro-A10	-17.937	-21.653	0.000	-39.590
m-Fluoro-A10	-17.787	-21.653	0.000	-39.440
A10	-17.686	-21.688	0.000	-39.351
o-Difluoro-A10	-14.313	-22.855	0.000	-37.168
o-Fluorochloro-A10	-12.613	-22.152	0.000	-34.765

The energy of the p-OH-A10/DNA complex was significantly lower than the A10/DNA complex. Analogs of A10 including p-OH A10 were synthesized and tested for antineoplastic activity in a variety of human, rat and mouse tumor cell lines. The specific cell lines included: rat Nb2 lymphoma cells which require the hormone prolactin for growth; YAK mouse lymphoma cells; K562 human erythrocytic leukemia cells; MCF7 human breast cancer cells and T24 human bladder cancer cells. P-OH-A10 possessed up to 90% greater activity than A10. Analogs which did not bind both DNA strands were either equivalent to or less potent than A10 in activity. A summary of the results with the percent fit of the analogs into DNA and the respective biological activities normalized to100% for A10 is provided in the table below.

RELATIVE FIT OF ANALOGS INTO DNA COMPARED TO BIOLOGICAL POTENCY

		Cell Line Tested				
Compound	% Fit into DNA	Nb2	YAK	K562	MCF7	T24
p-OH-A10	164	313	1649	1350	1160	155
o-Fluoro-A10	102	93	96	105	----	----
p-Fluoro-A10	101	85	110	106	----	----
m-Fluoro-A10	100	114	112	118	----	----
A10	100	100	100	100	100	100
o-Difluoro-A10	94	71	----	----	----	----
o-Fluorochloro-A10	88	114	122	111	----	----

In conclusion, our molecular modeling approach was used to develop new A10 analogs. As measured by energy calculations, degree of fit into DNA was found to correlate with predicted biological potency. Namely, the best fitting analog designed by the technology, p-OH A10, proved to be the most active compound. This investigation coupled with the extensive studies previously conducted on A10 indicate that P-OH-A10 may be a promising new antitumor agent.

We wish to acknowledge Dr. T. G. Muldoon who collaborated on this project up until his death in 1989 as well as participants in various stages in the generation of the computer modeling laboratory include M. Bridges, J. Wood, K. Nixon and Dr. F. Witham.

Synergistic Effect of Antineoplaston A5 and Retinoic Acid on the Induction of Human Promyelocytic leukemia cell line HL-60

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Leukemia cell line HL-60 differentiates in the presence of retinoic acid (RA) and Antineoplaston A5 (A5). Data reported here are based on NBT-positive fraction of HL-60 cells at 96 h of exposure. Under normal conditions, the cell line has 10% NBT-positive cells. In the presence of .15 μg/ml and .3 μg/ml RA, the fraction of NBT-positive cells increased to 32.00 ± 2.71% (A) and 37.50 ± 3.70% (B). While under treatment with 0.15 and 0.3 mg/ml A5, NBT-positive cells were 29.50 ± 2.89% (C) and 42.25 ± 5.62% (D). After exposure to combination of .15 μg/ml RA and 0.15 mg/ml A5, the NBT-positive reached 71.00 ± 6.83% (E). The differences between A vs. E, B vs. E and C vs. E are highly significant at $P<0.001$ and between D vs. E also significant at $P<0.01$. The results clearly indicate that synergistic effect exists between RA and A5.

Due to neoplastic cells heterogenicity, it is very unlikely that treating cancer with a single agent would lead to total eradication of all tumor cell subpopulations in a patient (1). Therefore, a combination of chemotherapeutic agents is preferred for treatment of neoplastic diseases. An ideal combination therapy employs agents that work synergistically.

Retinoic acid has been shown to induce cell differentiation in human promyelocytic leukemia HL-60 cells (2). Recently, we have documented that Antineoplaston A5 also induced HL-60 cells to undergo terminal cell differentiation (3). In addition, retinoic acid also has been shown to enhance HL-60 cell differentiation in response to dibutyryl cAMP and lymphokines (4,5). In this communication we report the synergistic effect of RA and A5 on the induction of HL-60 cells to undergo terminal differentiation.

Data are based on NBT-positive fraction of HL-60 cells at 96 hours of exposing to various treatments. Under normal growth condition, the cell line has 10% NBT-positive cells. In the presence of 0.15 μg/ml and 0.3 μg/ml RA, the percentage of NBT positive cells increases to 32.0 + 2.71 (A) and 37.5 + 3.71 (B) respectively, while at 0.15 mg/ml and 0.3 mg/ml A5, the percentage of NBT-positive cells are 29.5 ± 2.89 (C) and 42.3 ± 5.62 (D). However, in the combination of 0.15 μg/ml RA plus 0.15 mg/ml A5, the percentage

of NBT-positive cells increases to 71.0 + 6.83 (E).

The differences between A vs. E, B vs. E and C vs. E are highly significant at $P<0.001$ and the difference between D vs. E is also significant at $P<0.01$. The fact that the combined single dose (1x) of both agents yield significantly higher NBT-positive cells than either agent treated at double doses (2x) clearly indicated that a synergistic effect exists between RA and A5.

Further evidences of synergistic effect between RA and A5 come from the experiment done on RA resistant variant of HL-60 line called HL-60 RA (2). This variant has 2% NBT-positive cells. In the presence of 0.375 μg/ml RA at 120 hours, the NBT-positive cells remain very low at 3.0 + 0, while in the presence of 0.3 mg/ml A5, the percentage of NBT-positive cells increases to 25.3 + 10.50. However, the combination of 0.375 μg/ml RA plus 0.3 mg/ml A5 brings the NBT-positive cells to 73.3 + 10.50%. This value is far exceeding the combined positive effects from both agents used separately. Our results suggest that a combination therapy of these two agents may bring better results in cancer treatment.

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The Inhibitory Effect of Combination Use of Anitineoplaston A-10 Injection with Small Dose of CDDP on Cell and Tumor Growth of Human Hepatoma

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The inhibitory effect of Antineoplaston A-10 injection and small dose of CDDP was tested in invitro, invivo settings with human hepatoma. The mode of action of A-10 injection on hepatoma as cytostatic until 6 mg/ml but became cytotoxic at higher concentration where CDDP was cytotoxic at lower concentration. The combination of A-10 injection and small dose of CDDP inhibited cell growth most in invitro study. Electron microscopic study confirmed well developed organelle in A-10 treated cell suggesting A-10 slows cell growth by maturing cell rather than damaging cell. Invitro study also confirmed the combination of A-10 injection with small dose of CDDP inhibited tumor growth most.

The inhibitory effect of the combination use of Antineoplaston A-10 injection (Na salts of phenylacetyglutamine and phenylacetylisoglutamine in the ratio of 4:1) and small dose of CDDP was tested in invitro and invivo settings. Human hepatoma cell line (KIM-1) was used for cell growth and for tumor growth studies.
In invitro study KIM-1 cells in CDDP group were exposed to 0.5, 1.0, 2.0 ug/ml of CDDP for one hour and rinsed and allowed to be incubated in the medium.
KIM-1 cells in A-10 group were incubated in the medium which contained 4, 6, 8 mg/ml of Antineoplaston A-10 injection. Medium were renewed every two days. Cells in combined group were treated with CDDP and A-10 injection. Electronmicroscopic study was performed on 5th and 9th day of incubation. The mode of inhibition of cell growth was quite different with A-10 injection from CDDP. A-10 injection was cytostatic until the concentration reached to 6 mg/ml and became cytotoxic at 8 mg/ml where CDDP was cytotoxic even at lower concentration.
Electronmicroscopic study confirmed well developed organelle such as mitochondria, rough surfased endoplasmic reticulum, goldi apparatus suggesting cell maturation in 4 mg/ml, 6 mg/ml A-10 injection treated cells. There were irregular nucleus, secondary lysosome in 8 mg/ml of A-10 injection treated cells. There were irregular nucleus loose intercellular joint, fat droplets in CDDP treated cells. Those finding would suggest Antineoplaston A-10 may slow cell growth by maturing cell rather than damaging cell. In invivo study, KIM-1 cells were implanted and then transplanted to athymic mice. Tumor weight was calculated by the formula of width x width x length/2.
CDDP was administered intraperitoneally one a week for 5 consecutive weeks. A-10 injection was administered intraperitoneally every day for the whole experimental period.
20 ug/ml of CDDP + 75 mg/ml A-10 injection showed significant inhibition of tumor growth after 33 days treatment where 60 ug/ml of CDDP and 75mg of A-10 injection did not show any significant inhibition between control. Mice body weight dropped in only 60 ug/ml of CDDP treated mice.

Those data would indicate that the combination use of A-10 injection and small dose of CDDP could potentiate antitumor effect and reduce the undesirable side effect from both agents.

The Effect of Antineoplaston A-10, AS2-1 on Brain Tumor

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Six cases of brain tumor (5:primary; 1:metastatic) treated with Antineoplaston A-10, AS2-1 were reported. Multiple brain metastases from breast cancer had been stable for 18 months. One tumor in the head of caudate decreased its size and another tumor reduced perifocal edema. Primary thalamic anaplastic astrocytoma (III) disappeared with combination use of AS2-1 and synchronization (radiation, ACNU, Vincristine, Interferon). But a metastasis in the IVth ventricle developed when the dose of AS2-1, A-10 decreased. Two other gliomas have been stable for 3 months with AS2-1. Two gliobastomas progressed diseases.

Antineoplastons are naturally occurring peptides and aminoacid derivatives which control neoplastic growth, firstly isolated from human urine by Dr.Bruzinski. Antineoplaston A-10 (phenylacetylaminoglutamine -2, 6-piperidinedione) is the first compound identified and synthesized. Antineoplaston A-10 Injection (Na salts of 4 : phenylacetylglutamine+1 : phenylacetylisoglutamine), Antineoplaston AS2-1 (1 : phenylacetylglutamine+4: phenylacetic acid) are degradation products when Antineoplaston A-10 is orally administered. Both A-10 Injection and AS2-1 have antitumor effect. Six cases of brain tumor (5 : primary, 1 : metastatic) were reviewed in this study.

Case 1. Multiple brain metastases

Fifty eight year-old female who had had superradical mastectomy for early stage breast cancer (invasive ductal carcinoma). Eight year later she developed solitary lung and multiple brain metastases. Metastatic tumors were located left temporal lobe (0.8x0.8cm), left frontal lobe (1.5x1.2cm) and right head of caudate (1.5x2.0cm). She refused conventional chemotherapy because she had had awful experience with it when she took it for prophylactic purpose. Continuous A-10 Injection infusion was started through IVH catheter for 30 days, and followed by daily administration of A-10 cap 8g/day. Tumor in the head of caudate reduced its size in 10 months. Tumor in temporal lobe reduced perifocal edema in 10 months. Tumor in lung and other part in brain did not change their size for 18 months and started to grow a little bit. No serious side effect was observed. She has been tolerating treatment very well.

Case 2. Primary thalamic anaplastic astrocytoma (III).

Thirty three year-old male was found to have the primary thalamic tumor. Emergent V-P shunt and biopsy was performed to relieve his hydrocephalus. AS2-1 8g/day started and 2 weeks later, followed by synchronization consist of radiation, Vincristine, ACNU. Tumor size decreased in one month confirmed by MRI and continued to decrease and became almost invisible on MRI 4 months later.

Since no trace of tumor was found in thallium scintigram, the dose of AS2-1 started to decrease.

No tumor was observed on MRI 7 months later but he developed sudden double vision, vertigo. A tumor in fourth ventricle appeared 10 months later. The dose of AS2-1 increased to 8g/day and synchronization was conducted. The tumor started to decrease its size again.

Case 3. Recurrence of glioblastoma.

Forty one year-old female, had had removal of glioblastoma, developed recurrence soon. Synchronization plus AS2-1 8g/day started. Symptomatic improvement was noted first but the disease was continuously progressing.

Case 4. Recurrent intraventricular dissemination of glioblastoma.

Thirty three year-old male, who had had right temporal lobectomy for glioblioblastoma multiforme, developed intraventricular dissemination one year later.

A-10 8g/day started with 300mg of Endoxan. Tumors had been stable for 4 months, but grew fast after he became unable to take A-10 or AS2-1 regularly.

Case 5. Brain stem glioma.

Eight year-old male with brain stem glioma developed hemiplegia. AS2-1 6g /day started. Tumor size has not changed in 3 months on MRI.

We feel Antineoplaston A-10, AS2-1 are quite useful mean for the brain tumor patient on maintenance therapy and could be very useful for reduction of size in combination of other agents.

Phase II Clinical Trials of Antineoplaston A10 and AS2-1 Infusions in Astrocytoma

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The purpose of this study is the determination of effectiveness of Antineoplastons A10 (A10) and AS2-1 (AS) in control of astrocytoma in a limited number of patients. A10 and AS infusions were administered to 20 patients diagnosed with advanced astrocytomas. 18 patients were classified as stage III and IV and two patients were stage IIB. The infusions were administered from 67 to 706 days nightly. Dosage levels of A10 were from 0.5 to 1.3 g/kg/24h and AS from 0.2 to 0.5 g/kg/24h. Most of the patients had no adverse reactions. At the end of the study responses of four patients were classified as complete remission, two as partial remission, ten as objective stabilization and four as progressive disease. The best results were obtained in grade 3 astrocytoma. Surviving patients have now been followed for over three years.

Clinical observations during Phase I trials of Antineoplastons A10 (A10) and AS2-1 (AS) indicated objective responses in the treatment of primary malignant brain tumors, which prompted us to select these formulations for Phase II trials (1-3). In the present study, A10 and AS infusions were administered to 20 patients diagnosed with advanced astrocytomas. Most of the patients were 26 to 47 years old and began the treatment in the spring and summer of 1988. Eighteen patients were diagnosed with astrocytoma, stages III and IV. Two patients were stage IIB astrocytoma. Fifteen patients had grade 3 tumors, three patients had grade 2 and one patient had grade 1. One patient was initially diagnosed with grade 1 tumor, but subsequently the aggressive course of the disease suggested grade 3. All patients had diagnosis confirmed by biopsy. Most of the patients had performance status from 50 to 60 (Karnofsky).

Only one patient seemed to have benefit from his previous conventional treatment when beginning this program. Eighteen patients had clear progression of the disease after previous conventional treatment.

Infusions of A10 and AS (100 mg/ml) were administered through a subclavian vein catheter daily at 100 ml/h during the night. The shortest duration of the treatment was 67 days and the longest was 706 days. Most of the patients received from three to ten months of treatment. A10 was administered at dosage levels from 0.5 to 1.3 g/kg/24 h with most patients taking from 0.8 to 1.2 g/kg/24h. AS was given at dosages ranging from 0.2 to 0.5 g/kg/24h. The majority of patients were taking from 0.3 to 0.4 g/kg/24h of AS.

In addition to A10 and AS, common prescription drugs, except for chemotherapeutics, were given to some patients due to their advanced stage of cancer. Most of the patients continued the treatment with A10 and AS without any adverse reactions. Five patients had mild side effects occurring sporadically during the course of treatment. They included febrile reaction in two patients, slight decrease of platelet count in one patient and slight decrease of WBC in three patients. Side effects were very mild and they did not have any impact on the continuation of treatment.

Four patients, WP, SB, WT and BR obtained complete remission as a result of the treatment. Two of them were diagnosed with stage IV disease, one with stage IIIA and one with IIB.

The patient WP is a 36 year old female who was diagnosed with anaplastic astrocytoma of the brain stem, stage IV (G3T4M0). She was initially treated with radiation therapy, but had clear progression of the disease before starting on the program. Her complete remission was documented after approximately six months of treatment. She continues to be in complete remission 30 months later.

The patient SB (33 year old female) was diagnosed with anaplastic astrocytoma, stage

IIIA (G3T2MO), and was initially treated by two subtotal resections, radiation therapy and a single dose of 100 mg of CCNU. She had an aggravation of her symptoms before beginning the treatment with A10 and AS. Once on treatment, the patient obtained complete remission after approximately seven months. Currently, 27 months later, she continues to be in complete remission and is off the treatment program.

The patient BR was diagnosed with astrocytoma, stage IV (G3T4MO). Initially he had partial tumor resection, radiation therapy, chemotherapy with BCNU and repeated tumor resection. The disease continued to progress. After approximately seven months of treatment with A10 and AS, he was classified in complete remission. After discontinuation he developed recurrence and died 20 months later.

The patient WT was a 32 year old white male who was diagnosed with oligoastrocytoma, stage IIB (G2T1MO). This patient received radiation therapy which resulted in some symptomatic and objective improvement more than six months before beginning the treatment with A10 and AS. Once on A10 and AS his complete remission was documented after 54 days. Five months later, after discontinuation of the treatment, he developed recurrence and died nine months after obtaining complete remission.

It is possible that in one of these cases (WT), there was a beneficial effect from radiation therapy administered over six months before the treatment with antineoplastons.

Two patients, MP and YL, obtained partial remission as the result of the treatment with A10 and AS. MP is a seven year old boy who was diagnosed with astrocytoma, stage IV (G1T4MO). The tumor was located in the suprasellar region. This patient received no conventional treatment prior to therapy with antineoplastons. His response was slow with continuous tumor reduction. After approximately 22 months of treatment he accomplished partial remission. At present, he is on a different antineoplaston protocol.

The patient YL was a 15 year old female diagnosed with anaplastic astrocytoma, stage IV (G3T4MO). In spite of her initial treatment with radiation therapy, there was progression of the disease. She subsequently underwent subtotal resection of the tumor and was placed on chemotherapy with BCNU. Unfortunately, she had further progression of the disease. After approximately 8 months of treatment with A10 and AS, her response was classified as partial remission. Four months later she developed hemorrhage from the necrotic tumor and passed away.

Ten patients obtained objective stabilization of the disease. Seven of these patients had marked decrease of the tumor size. Three patients from this group were switched to another treatment program with antineoplastons. Two of them are now in complete remission and one in partial remission. Five patients discontinued the program against medical advice. Two of them are known to be alive, three others expired. Two patients expired due to complications not associated with the treatment and activity of the disease.

Four patients had increasing disease during the course of the treatment with A10 and AS. One of them had progression of the disease after initial objective improvement. Two of these patients passed away.

The results of this trial indicate anticancer activity of A10 and AS in the treatment of astrocytoma. Only 20% the of patients did not benefit from the treatment whereas the majority went into complete and partial remission or had objective stabilization. The patients who responded best have now been in complete remission for approximately 30 months. Anaplastic astrocytoma responds the fastest, whereas regression of low grade astrocytoma takes a much longer time.

The treatment schedule with A10 and AS is completely different from that of standard chemotherapy. A10 and AS are differentiation inducers which require daily administration over an extended period of time (4,5). The study indicates the importance of taking the treatment for a sufficient period of time. Two patients who initially obtained complete remission developed recurrence of the disease when the treatment was discontinued too soon. Some patients will benefit more when the infusions are given continuously. Three patients whose disease had been stabilized on this protocol of nightly administration went into complete and partial remissions when they were switched to continous infusions. The results of this study will be compared with the effectiveness of the current protocol for continous infusions and the best modality will be selected for Phase III trials.

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